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AND THERAPEUTIC COMPOSITIONS AND METHODS FOR INHIBITION  
AND TREATMENT OF TUMOR ASSOCIATED ANGIOGENESIS

~~ANGIOGENESIS INHIBITORY ANTIBODIES~~

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5 FIELD OF THE INVENTION

This invention relates to angiogenesis inhibitory antibodies, and to the use thereof in the inhibition of angiogenesis, particularly angiogenesis associated with the growth of solid tumours, with proliferative retinopathies, and with certain inflammatory diseases.

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BACKGROUND TO THE INVENTION

The circulatory system represents an extensive, branching, network of blood vessels which is essential for the supply of oxygen and nutrients to tissues and for the removal of byproducts of metabolism. In adults the development of new blood vessels or "angiogenesis" rarely occurs except during wound healing or as a result of a number of pathological situations termed "angiogenesis-dependent diseases"<sup>(1,2)</sup>. The most important of these is the angiogenesis associated with the growth of solid tumours and with proliferative retinopathies. Angiogenesis may also play an important role in rheumatoid arthritis and psoriasis.

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Angiogenesis inhibitors can, therefore, be of considerable value in the treatment of angiogenesis-dependent diseases. For example, in the case of solid tumours, the development of a blood supply is essential for the growth and survival of the tumour. Thus, inhibition of angiogenesis can provide a highly 20 selective means of inducing tumour regression. Similarly, angiogenesis inhibitors may be used to prevent the blindness associated with proliferative diabetic retinopathy, one of the major complications of diabetes.

In work leading to the present invention, monoclonal antibodies (mAbs) 25 have been developed against proliferating/angiogenic human endothelial cells which can be used either to directly inhibit angiogenesis or to target cytotoxic drugs or radioisotope labels to sites of angiogenesis. Since angiogenesis does

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not occur in adults, except following tissue injury, such mAbs can be remarkably specific. Furthermore, unlike other lines of research which have produced cancer cell-specific mAbs to target cytotoxic drugs to tumours, the present invention is directed to producing mAbs against host antigens. This approach has the major  
5 advantage that generation of "resistant" variants of the tumour cannot occur and, in theory, one mAb could be used to treat all solid tumours. An additional advantage is that endothelial cells, by virtue of their vascular location, are very accessible to mAbs in the circulation.

#### 10 SUMMARY OF THE INVENTION

According to the present invention, there are provided antibodies, including monoclonal antibodies, specific for proliferating/angiogenic human endothelial cells.

15 More particularly, the present invention provides antibodies, including monoclonal antibodies, specific for proliferating/angiogenic human umbilical vein endothelial cells (HUVEC) or human umbilical artery endothelial cells (HUAEC).

20 This invention also extends to hybridoma cell lines producing the monoclonal antibodies as described above, which may be produced by methods well known to persons skilled in this field.

25 As previously described, the antibodies in accordance with the invention may be used alone as an anti-angiogenesis agent in the treatment of angiogenesis-dependent disease in a patient.

In another aspect, the present invention provides an antibody-conjugate comprising an antibody specific for proliferating/angiogenic human endothelial cells, having a toxin material or label conjugated thereto.

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The toxin material may, for example, be a cytotoxic drug or other cytotoxic material, however other toxin materials well known to persons skilled in this art

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may also be incorporated in the antibody-conjugate of this aspect of the invention. The label may be a radioisotope. Suitable toxin materials include, by way of example, ricin A chain, diphtheria toxin, *Pseudomonas* exotoxin A and idarubicin. A suitable radiolabel is technetium -99m. Coupling of various toxins to monoclonal 5 antibodies may be effected by known methods<sup>(3,4,5,6)</sup>. Similarly, the preparation of a conjugate with a radiolabel may use known methods<sup>(7)</sup>.

In yet another aspect, the invention provides a composition, particularly a therapeutic composition for inhibition of angiogenesis or for treatment of 10 angiogenesis-dependent disease, comprising an antibody or antibody-conjugate as broadly described above, together with a pharmaceutically acceptable carrier or diluent.

The present invention also extends to a method for inhibition of 15 angiogenesis in a patient, for example angiogenesis associated with the growth of solid tumours or with proliferative retinopathies, which comprises administration to said patient of an inhibition-effective amount of an antibody or antibody-conjugate as broadly described above.

20 In another aspect, this invention provides a method for treatment of angiogenesis-dependent disease in a patient, which comprises administration to said patient of a therapeutic-effective amount of an antibody or antibody-conjugate as broadly described above.

25 Administration of the antibody or antibody-conjugate may be by any suitable route. Preferably, the administration to the patient is parenterally, for example, by injection.

#### DETAILED DESCRIPTION OF THE INVENTION

30 In accordance with one embodiment of this invention, there have been developed monoclonal antibodies (mAbs) specific for proliferating/angiogenic endothelial cells. The major use of these mAbs is to simply inhibit angiogenesis,

5 although if desired the mAbs can be used to target cytotoxic drugs or labels to angiogenic sites. In the case of tumours, this approach has the major advantages of tumour specificity, minimal side-effects, and little chance of "resistant" tumour variants arising. Furthermore, these mAbs provide a single therapeutic agent that can be used for all solid tumours, regardless of type and tissue location, and inhibition of angiogenesis in the solid tumours can result in tumour regression.

The initial experimental approach has been to raise murine mAbs against proliferating/angiogenic human umbilical vein endothelial cells (HUVEC). Resultant mAbs have been screened initially for HUVEC reactivity and, subsequently, mAbs have been eliminated which react with other human cell lines, e.g. human melanoma cell lines. Finally, endothelial specific mAbs have been identified which fail to react with freshly isolated, non-proliferating/non-angiogenic human endothelial cells. Using this approach, it has been clearly established that mAbs can be obtained which are specific for proliferating/angiogenic human endothelial cells.

**BRIEF DESCRIPTION OF THE DRAWING**

Figure 1 shows binding of mAbs to proliferating/angiogenic and resting (non-proliferating/non-angiogenic) human umbilical vein endothelial cells (HUVEC) as detected by immunofluorescence flow cytometry. CONT refers to HUVEC not incubated with mAbs, 20G5 is a HUVEC-specific mAb which reacts with both proliferating/angiogenic and resting HUVEC and 9B11 is a HUVEC-specific mAb which only reacts with proliferating/angiogenic HUVEC.

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Further details of the present invention will be apparent from the following detailed description of the production of endothelial specific mAbs in accordance with the invention.

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## EXAMPLE

### A. Materials and Methods

#### Cells

5 Human umbilical vein (HUVEC) and artery (HUAEC) endothelial cells were prepared from human umbilical cords by the method of Jaffe<sup>(8)</sup> and cultured in Medium 199 supplemented with 20% foetal calf serum (FCS), L-glutamine, antibiotics, 130 ug/ml heparin and 1.2 mg/ml endothelial cell growth supplement (Sigma). HUVEC were used for mAb binding studies between passages 2 and 7.

10 Human tumour cell lines (e.g. MM-170 melanoma, K562 erythroleukaemia) were cultured in RPMI-1640/10% FCS. Mononuclear cells (lymphocytes and monocytes) and neutrophils were simultaneously isolated from human peripheral blood by centrifugation of diluted blood on Polymorphprep<sup>TM</sup> (Nycomed. Pharma A.S., Oslo, Norway). Red cells and platelets were isolated by differential

15 centrifugation from citrated human blood.

#### Production of Hybridomas

BALB/c mice were immunised, i.p., 3-4 times at 2-4 weekly intervals with 15x10<sup>6</sup> HUVEC in PBS and challenged 3 days prior to spleen cell removal with

20 15x10<sup>6</sup> HUVEC. A spleen cell suspension was prepared, fused with the myeloma NS1/1.AG4.1 and hybridomas grown up and cloned as described previously<sup>(9)</sup>. To improve hybridoma growth and cloning efficiencies 10% endothelial cell conditioned medium (HUVEC or bovine corneal EC) was included in culture media.

#### 25 mAb Screening Assays.

Initially hybridoma culture supernatants were tested for reactivity with HUVEC by immunofluorescence flow cytometry. Briefly, HUVEC (5x10<sup>4</sup>) were incubated (30 min, 4°C) with undiluted hybridoma supernatant, washed and incubated with FITC-sheep F(ab')<sub>2</sub> anti-mouse Ig(100µg/ml). Following final 30 washing HUVEC were examined for mAb binding by analysis on a Becton-Dickinson FACScan. Positive hybridoma supernatants were then screened on the human melanoma cell line MM-170 to eliminate non-endothelial specific mAbs.

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Endothelial specificity was further confirmed by screening mAbs on a panel of human tumour cell lines and human lymphocytes, monocytes, neutrophils, red cells and platelets. Finally, specificity for proliferating HUVEC was established by screening hybridoma supernatants on freshly isolated (non-cultured) HUVEC.

5    Hybridomas which were positive on proliferating HUVEC but negative on freshly isolated HUVEC were cloned<sup>(9)</sup> for further study. A number of hybridomas (e.g. 20G5) which were endothelial-specific but not proliferation/angiogenesis-specific were also cloned.

#### 10    HUVEC Proliferation Assay

Assays were performed in 96 well, flat bottom, microplates coated with 0.1% gelatin and containing  $2.5 \times 10^4$  HUVEC/well in 150 $\mu$ l of culture medium. After 24hr culture cells were pulsed with  $^3$ H-thymidine for a further 24hr and  $^3$ H-thymidine incorporation assessed in washed and harvested cells using a Titertek 530 cell harvester (Flow Labs). In mAb blocking experiments 50  $\mu$ l/well of hybridoma supernatant was added at the commencement of the cultures with supernatant from a hybridoma which does not react with HUVEC being used as a negative control.

#### 20    B.    Results

##### Production of mAbs Specific for Proliferating/Angiogenic Endothelial Cells

Table 1 shows that mAbs can be obtained which are specific for proliferating/angiogenic human endothelial cells.

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TABLE 1 Production of Endothelial Specific Monoclonal Antibodies (mAbs).

Hybridomas	Number	
	Fusion #1	Fusion #2
5 Total screened	1196	660
Proliferating HUVEC positive	811	276
Proliferating HUVEC specific	541 <sup>a</sup>	102 <sup>b</sup>
Non-proliferating (resting) HUVEC negative	25 <sup>c</sup>	17 <sup>c</sup>

10 a Hybridomas not reactive with the human melanoma cell line MM-170.

b Hybridomas not reactive with human MM170 cell line, U937 monocytic cell line, lymphocytes, neutrophils, monocytes, red cells and platelets.

15 c Hybridomas not reactive with endothelial cells freshly isolated from the human umbilical cord, i.e. endothelial cells "non-proliferating" or "resting".

20 HUVEC = Human umbilical vein endothelial cells.

In the first fusion of 1196 hybridomas screened, 811 reacted with proliferating/angiogenic endothelial cells of which 541 were proliferating/angiogenic endothelial cell specific, i.e. failed to react with other

25 proliferating human cell lines such as the human melanoma line MM-170. Of particular importance was the fact that 25 of the 541 hybridomas specific for proliferating/angiogenic human endothelial cells failed to react with non-proliferating/non-angiogenic (freshly isolated) endothelial cells. Thus, 4.6% of hybridomas produce mAbs which are proliferation/angiogenesis specific, a clear

30 validation of the approach being used. A similar result was obtained in a second fusion where 16.6% of the HUVEC-specific mAbs were angiogenesis specific. A typical example of the results obtained with a proliferation/angiogenesis-specific (9B11) and a proliferation/angiogenesis non-specific (20G5) mAb is depicted in Fig.1 as revealed by immunofluorescence flow cytometry.

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Table 2. Reactivity Pattern of Some Cloned Monoclonal Antibodies Against Human Endothelial Cells

Human Cells	mAb Clones					
	9D9 (IgM)	12E5 (IgM)	10A5 (IgM)	14G11 (IgG1)	21F10 (IgM)	20G5 (IgM)
Proliferating HUVEC	+	+	+	+	+	+
Resting HUVEC	-	-	-	-	-	+
Proliferating HUAEC	+	+	+	+	+	+
K562 erythroleukaemia	-	-	+	+	+	-
MM170 melanoma	-	±	+	+	+	-
PE.01 ovarian carcinoma	-	-	+	+	+	-
COLO397 colonic carcinoma	-	-	+	+	+	-
KJD keratinocyte carcinoma	-	-	+	+	+	-
MT2 B lymphoma	-	-	+	+	+	+
Molt 4 T lymphoma	-	-	+	+	+	+
U937 (monocytic)	-	-	+	+	+	-
Lymphocytes	-	-	+	-	-	+
Neutrophils	-	±	±	-	-	+
Monocytes	-	+	+	±	-	+
RBC	-	-	-	-	-	-
Platelets	±	-	±	+	-	+
Fibroblasts	-	-	±	±	-	-

HUVEC = human umbilical vein endothelial cells.

HUAEC = human umbilical artery endothelial cells.

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Table 2 presents detailed specificity analysis of six cloned mAbs, which were HUVEC reactive, as examples. One mAb (20G5) is a control which reacts with both resting and proliferating/angiogenic endothelial cells and is probably 5 specific for the CD31 antigen. The remaining five mAbs react with proliferating/angiogenic but not resting endothelial cells. Three of these mAbs (10A5, 14G11, 21F10) react with many other proliferating cell types. The remaining two clones (9D9 and 12E5) exhibit considerable specificity for 10 proliferating/angiogenic endothelial cells, 9D9 being the mAb with the greatest specificity, only exhibiting a weak reaction with platelets.

The 9D9 mAb reacts with proliferating/angiogenic venular or arterial endothelial cells but not non-proliferating (resting) endothelial cells (Table 2). Subsequent studies showed that the 9D9 antigen appears on cultured HUVECS 15 within 24 hr of culture and persists on HUVEC cultured for many passages, i.e. ten passages over a period of two months. The 9D9 antigen also appears on HUVEC whether they are cultured in 20% FCS + bovine growth supplement or 20% human serum, indicating that the 9D9 antigen is not derived from culture medium components.

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#### Effect of mAbs on Endothelial Cell Proliferation.

When some of the proliferation-specific mAbs were added to proliferating HUVEC *in vitro* it was found that some of the mAbs could directly inhibit HUVEC proliferation. The results of a typical experiment are present in Table 3.

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TABLE 3 Inhibition of HUVEC Proliferation by mAbs Specific for Proliferating/Angiogenic Endothelial Cells.

mAb	Specificity	$^3\text{H}$ -Thymidine Incorporation* (cpm)	Response % Control
9B9	Non-reactive	7779±1420	100
5 20G5	HUVEC	6806±1290	87.5
1D5	Proliferating HUVEC**	1256±110	16.1
8G4	Proliferating HUVEC**	1857±38	23.9
16C6	Proliferating HUVEC**	1767±175	22.7
19D4	Proliferating HUVEC**	7530±753	96.8

10 \* HUVEC cultured in proliferation assay with dialyzed hybridoma supernatants containing mAbs. Proliferation measured 24-48 hr following culture initiation and represents mean  $\pm$  standard error of three determinations.

15 \*\* mAbs only react with proliferating/angiogenic (not resting) HUVEC.

20 Of the four proliferation/angiogenesis-specific mAbs tested, three (1D5, 8G4 and 16C6) inhibited HUVEC proliferation by approx. 75-85% as measured by  $^3\text{H}$ -thymidine incorporation. In contrast, one proliferation/angiogenesis-specific mAb (19D4) and 20G5, a mAb which reacts with both proliferating and non-proliferating HUVEC, had no significant effect on HUVEC proliferation. The mAb 9B9, which does not react with HUVEC, was used as the negative control in this experiment.

25 These data strongly suggest that some of the proliferation/angiogenesis-specific mAbs may directly inhibit angiogenesis, thus bypassing the need for cytotoxic drug-mAb conjugates. It should be emphasised that the data presented in Table 2 were obtained with hybridoma supernatants and not with purified and concentrated mAb preparations.

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